PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C12N 15/56, 9/24, A23K 1/165
A21D 8/04, D21C 9/10

A1

(11) International Publication Number: WO 93/25693

(43) International Publication Date: 23 December 1993 (23.12.93)

(21) International Application Number: PCT/GB93/01283

(22) International Filing Date: 17 June 1993 (17.06.93)

(30) Priority data:
PL 2985 17 June 1992 (17.06.92) AU

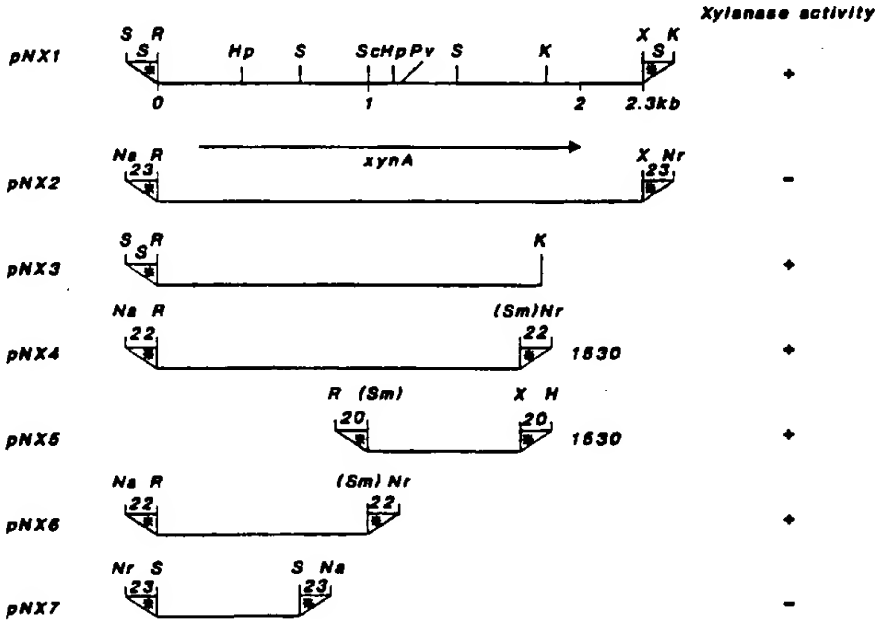
(71)(72) Applicants and Inventors: HAZLEWOOD, Geoffrey, Peter [GB/GB]; 109A Duchess Drive, Newmarket, Suffolk CB8 2AL (GB). GILBERT, Harry, John [GB/GB]; 16 Kells Gardens, Low Fell, Gateshead, Tyne and Wear NE9 5XS (GB).

(74) Agents: SHEARD, Andrew, Gregory et al.; Kilburn & Strode, 30 John Street, London WC1N 2DD (GB).

(81) Designated States: AU, BB, BG, BR, CA, CZ, FI, HU, JP, KP, KR, KZ, LK, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SK, UA, US, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: RECOMBINANT XYLANASES



(57) Abstract

Recombinant xylanases are derived from anaerobic fungi, particularly *Neocallimastix patriciarum*. The enzymes are highly specific for xylans and have industrial value, particularly in the pulp and paper industries. Certain truncated forms of the enzymes, and enzymes encoded by truncated DNA sequences, are preferred for their high expression levels.

In the animal feed industry, the use of enzyme supplementation to improve feed for chicks was reported as early as 1957. More recent results suggest that, in certain grains such as wheat, and particularly rye, it is the pentosans in the endosperm that are mainly responsible for poor nutrient uptake and sticky droppings from the chicks. Both problems appear to result from the high viscosity of the undigested pentosans. This hampers the diffusion of nutrients and binds water to make excreta watery. The problems can be alleviated using xylanase preparations. Xylanase action can improve both the weight gain of chicks and their feed conversion efficiency. It appears that xylanase supplementation could be used to improve the nutritional value of rye, so as to promote the use of this grain in chick feed. The effectiveness of this treatment may be dependent on the variety of rye. The invention provides the use of xylanase in chick feed and grain for these purposes.

In the pulp industry, dissolving pulps are purified celluloses used for making viscose rayons, cellulose esters and cellulose ethers. They are derived from prehydrolysed kraft pulps or acid sulphate pulps. Their processing is characterised by the derivatisation of the cellulose at one stage, the derivative being soluble in common solvents and thus permitting the formation of fibres, films and plastics. Impurities in the cellulose hamper derivatisation and thus lead to insolubles that block orifices in sprayers or form defects in the final product. Furthermore, certain xylan impurities can lead to colour, haze and thermal instability in acetate products. Xylanases may thus have a role to play in removing impurities, and the use of xylanases described herein for this purpose is comprehended within the invention.

The prebleaching of kraft pulp using cellulase-free xylanase has been identified as one of the biotechnologies most likely to be accepted in the pulp and paper industry in the near future, but only if suitable xylanases become available. The

that the mRNA degrading activity of RNase E is the rate limiting step in protein synthesis, and that RNase E has a preference for AT-rich regions of mRNA. It is possible to further increase its expression level in *E. coli* by using a stronger promoter, such as Bacteriophage T_7 promoter.

Recombinant xylanase A (XYLA) purified from *Escherichia coli* harbouring *xyn*A, had an M_r, of 53000 and hydrolysed oat spelt xylan to xylobiose and xylose. The enzyme did not hydrolyse any cellulosic substrates. The nucleotide sequence of *xyn*A revealed a single open reading frame of 1821 bp coding for a protein of M_r 66192. The predicted primary structure of XYLA comprised of an N-terminal signal peptide followed by a 225 amino acid repeated sequence, which was separated from a tandem 40 residue C-terminal repeat by a threonine/proline linker sequence. The large N-terminal reiterated regions consisted of distinct catalytic domains which displayed similar substrate specificities to the full length enzyme.

Xylanases in accordance with the invention have a number of applications in the food, feed, and pulp and paper industries. The use of xylanases described herein in these industries is included within the scope of the invention.

Dealing first with the food industry, certain properties of dough and its resultant baked products are dependent on the pentosan and starch content of the flour used. These properties include the texture, volume and staling of bread. The use of xylanase could modify baked products to provide goods of potential commercial value. Among the properties that can be modified by xylanase treatment is the specific volume of bread. The increase in specific volume is enhanced further when amylase is added in combination with xylanase. One of the factors contributing to this effect is the water-binding capacity of carbohydrates. The invention provides dough including a xylanase as described herein.

kraft (also known as alkaline or sulphate) process has become the predominant pulping technology in Canada because it produces strong wood fibres and because the chemicals used are recovered and recycled. Kraft pulps, particularly those derived from softwoods, are relatively difficult to bleach. A sequence of stages using elemental chlorine and chlorine-containing compounds is traditionally required to bleach these pulps effectively to the desired full brightness of ~90%. The bleaching process, particularly when using elemental chlorine, products chloro-organics that have traditionally been discharged from the bleach plant with the waste water. However, both public demand and legislated regulations are presently pressurizing pulp mills to reduce or eliminate the emission of these pollutants. The pulp and paper industry is considering the implementation of various alternative technologies in order to reduce the environmental impact of its mills. These options include xylanase prebleaching of kraft pulp. Xylanases in accordance with the present invention are particularly well suited to this purpose.

It is believed that the xylanases of the present invention are particularly applicable to the paper and pulp industry. While it is appreciated that the use of enzymes will never replace chemicals completely, there is pressure being exerted by those concerned with the environment to reduce the use of chemicals. There are also practical reasons for reducing the use of chemicals in the paper and pulp industry.

Pulping plants usually generate their own supplies of chlorine and chlorine dioxide on site, and this can limit capacity as well as being potentially hazardous. Treating the paper pulp (eg kraft pulp) to remove lignin involves the use of chlorine, NaOH, H_2O_2 and chlorine dioxide. Sandoz in the USA have conducted practical trials using their CARTAZYME product, which is a fungal xylanase (crude), active at 30-55°C, pH 3 to 5, and contains 2 xylanases, and have found that a 25-33% reduction in chlorine is possible using 1U xylanase/gm pulp. Also the product is brighter than when chemicals alone are used. Another advantage of the xylanase

is that it is specific whereas chemicals can attack the cellulose at low lignin contents, leading to reduced fibre strength and other undesirable physical characteristics. It is therefore clear that xylanases could become more important in pulp bleaching and recombinant ones particularly so because of their specificity and high yield. It is believed that lignin is bonded to hemicellulose, and if the hemicellulose (xylan) is depolymerised the lignin may be partially disassociated from cellulose and subsequently washed out. At present, however, some chemical treatment may still be necessary. The main points about xylanase of the present invention, with respect to commercial use, are (i) its very high specific activity and high level of expression would make it economical to produce on a large scale and (ii) its lack of cellulase activity make it particularly useful where it is necessary to remove xylan specifically as applied to the paper making and textile industry.

It is also believed that the xylanase of the invention could find a valuable application in the sugar industry and in relation to the treatment of bagasse or other products containing xylan for more efficient disposal.

It was previously mentioned that the protein sequence of XYLA and the DNA sequence of *xyn*A were made available on 5 May 1992 on the EMBL database under accession number X65526. This availability may not constitute effective prior art in the jurisdictions of all of the states designated in this application. For those jurisdictions where the EMBL database entry does not constitute effective prior art, notice is hereby given that the invention is and will be defined more broadly than as indicated above. In particular, the invention may then be seen to reside in the following further aspects:

a xylanase which has at least one catalytic domain which is substantially homologous with a xylanase of an anaerobic fungus; the xylanase may be a full length natural xylanase of an anaerobic fungus; and